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(54) Title: RECOMBINANT PORCINE ADENOVIRUS VECTOR

(57) Abstract

This invention relates to a recombinant vector including a recombinant porcine adenovirus, stably incorporating and capable of expression of at least one heterologous nucleotide sequence. The nucleotide sequence is preferably one which encodes an antigenic determinant of Hog Cholera Virus or Pseudorabies virus. The further invention relates to a method of production of recombinant vectors, to methods of preparation of vaccines based on the vectors, to administration strategies and to methods of protecting pigs from disease.

RECOMBINANT PORCINE ADENOVIRUS VECTOR

FIELD OF INVENTION

This invention relates to delivery vectors for antigen producing genes (heterologous gene sequences or fragments thereof) used to generate immune responses in commercial pigs susceptible to decimation by disease. Such vectors are especially useful for the preparation of vaccines which can be easily administered on a large scale to protect pigs against disease. This invention also relates to a method of production of suitable delivery vectors, to methods of preparation of vaccines based on the vectors, to administration strategies and to a method protecting pigs from disease.

BACKGROUND

The productivity of the intensive pig industry depends on the control of infectious diseases. Whilst diseases can be controlled in part by good hygiene and quarantine measures, the industry must still rely on vaccination to protect herds. In a commercial situation, the cost per animal is high in terms of feed and current disease control costs and therefore, the costs in disease prevention and control by any newly proposed vaccine must be cheap, effective and easy to deliver.

Conventionally, vaccines constituting live viral particles have been prepared by virus passage and selection of attenuated forms. Alternatively, killed vaccines were prepared from virulent viruses.

The most recent description of the use of viral vectors in the control of disease in pigs was the deletion mutant of pseudorabies virus for the control of Aujesky's disease. The use of a herpesvirus as a vector has the advantage of being able to stimulate a humoral and cell-mediated response, thus providing possible life long protection. Another advantage is the ability to insert other heterologous sequences in this vector, being expressed from a suitable promoter, to produce antigens for exposure to the animals immune system, thus protecting against two diseases. There are disadvantages of this system. Firstly, there is the issue of latency. Herpesviruses have the ability to intergrate into the neurons in ganglia for the life of the animal. It only requires a suitable stress on the animal to cause the reactivation of the virus and consequently full

The recombinant vector may comprise a live recombinant porcine adenovirus in which the virion structural proteins are unchanged from those in the native porcine adenovirus from which the recombinant porcine adenovirus is produced.

This invention is partially predicated on the discovery that there are non-essential regions in the porcine adenovirus genome which do not correspond to those characterised previously on other adenoviruses thus making this virus particularly suited to delivery of heterologous sequences.

This invention is also predicated on the discovery that the porcine adenovirus generates a prolonged response in pigs thus making it well suited as a vaccine vehicle. Furthermore, the existence of a number of serotypes specific to respiratory or gastrointestinal tracts, allows the selection of a vaccine vehicle suited to a target organ and the type of immune response required.

The invention is also predicated on the discovery that porcine adenovirus can package genomic DNA greater than the 105% rule for mammalian adenoviruses with intermediate size genomes and that the resultant packaged virions are stable in vitro and in vivo.

Adenoviruses are a large and diverse family, having been isolated from many living species, including man and other mammals as well as a variety of birds. As a result adenoviruses have been separated into at least two genera, the *Mastoadenoviridae* and the *Aviadenoviridae*, and more recently a third genera has been proposed, the *Atadenovirdae*, which includes some bovine and avian adenoviruses (egg drop syndrome) (Benkö and Harrach, Archives of Virology 143, 829-837, 1998).

Porcine adenoviruses are prevalent infectious agents of pigs and to date four distinct serotypes have been recognised (Adair and McFerran, 1976) and evidence for at least one more (Derbyshire *et al.*, 1975). Of the four serotypes found, three (serotypes 1 to 3) were isolated from the gastrointestinal tract while the fourth was recovered from the respiratory system. The porcine adenoviruses are considered to be a low pathogenic widespread agent and although isolations were made in general from diseased animals, it was most likely that the adenovirus was present only as a secondary infection. They have been

adenoviruses (Benkö *et al.*, 1990). A recent report on the serotype 4 PAV demonstrated that its genomic layout was also similar to that of the human adenoviruses in the area of the L4 and E3 regions (including the 33K and pVIII genes) even though the sequence homology was not as strong as may have been expected (Kleiboeker, 1994).

While choosing appropriate PAV for development as a live vectors to deliver vaccines to pigs, it is important to take into account the natural prevalence of serotypes. Those serotypes not commonly encountered in the field have an obvious advantages over those to which pigs are frequently exposed and to which they may have developed immunity.

A further consideration is the ability of the vector to remain active in the pig beyond the period which maternal antibodies in colostrum protect pigs immediately post-birth.

Other important considerations in choosing potential PAV vectors are pathogenicity and immunogenicity. Preferably live vector viruses should be highly infectious but non-pathogenic (or at least attenuated) such that they do not themselves adversely affect the target species.

The preferred candidates for vaccine vectors are non-pathogenic isolates of serotype 4 (respiratory) and serotype 3 (gastrointestinal). Serotype 3 has been chosen as the serotype of choice due to excellent growth abilities in continuous pig kidney cell lines. The isolation of other serotypes, which seems likely, may well alter this selection. It is notable that the more virulent strains produce a greater antibody response.

Heterologous nucleotide sequences which may be incorporated into non-essential regions of the viral genome and which may encode the antigenic determinants of infectious organisms against which the generation of antibodies or cell-mediated immunity is desirable may be those expressing antigenic determinants of intestinal infections caused by gastrointestinal viruses; for example rotavirus or parvovirus infections, or respiratory viruses, for example parainfluenza virus, or that of Japanese encephalitis.

Heterologous nucleotide sequences which may be incorporated include the antigenic determinants of the agents of:

sequences may be non-coding regions at the right terminal end of the genome at map units 97 to 99.5. Preferred non-coding regions include the early region (E3) of the PAV genome at map units 81-84.

The heterologous gene sequences may be associated with a promoter and leader sequence in order that the nucleotide sequence may be expressed in situ as efficiently as possible. Preferably the heterologous gene sequence is associated with the porcine adenoviral major late promoter and splice leader sequence. The mammalian adenovirus major late promoter lies near 16-17 map units on the adenovirus genetic map and contains a classical TATA sequence motif (Johnson, D.C., Ghosh-Chondhury, G., Smiley, J.R., Fallis, L. and Graham, F.L. (1988), Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology 164, 1-14).

The splice leader sequence of the porcine adenovirus serotype under consideration is a tripartite sequence spliced to the 5' end of the mRNA of all late genes.

The heterologous gene sequence may also be associated with a poly adenylation sequence.

Instead of the porcine adenoviral major late promoter, any other suitable eukaryotic promoter may be used. For example, those of SV40 virus, cytomegalovirus (CMV) or human adenovirus may be used.

Processing and poly adenylation signals other than those of porcine adenoviruses may also be considered, for example, that of SV40.

In a further aspect of the invention there is provided a recombinant vaccine for generating and/or optimising antibodies or cell-mediated immunity so as to provide or enhance protection against infection with an infectious organism in pigs, the vaccine including at least one recombinant porcine adenovirus vector stably incorporating at least one heterologous nucleotide sequence formulated with suitable carriers and excipients. Preferably the nucleotide sequence is capable of expression as an antigenic polypeptide or as an immuno-potentiator molecule. More preferably, the heterologous nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

for and/or express, an antigenic polypeptide and an immuno-potentiatormolecule. The nucleotide sequence is conveniently foreign to the host vector.

Even more preferably the nucleotide sequence is associated with promoter/leader and poly A sequences.

The form of administration may be that of an enteric coated dosage unit, an inoculum for intra-peritoneal, intramuscular or subcutaneous administration, an aerosol spray, by oral or intranasal application. Administration in the drinking water or in feed pellets is also possible.

In another aspect of the invention, there is provided a method of producing a porcine adenovirus vaccine vector which includes inserting into a porcine adenovirus at least one heterologous nucleotide sequence. Said heterologous nucleotide sequence is preferably capable of expression as an antigenic polypeptide although it may also be an immuno-potentiator molecule. More preferably, the nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

Preferably the antigenic polypeptide encoded by the at least one nucleotide sequence is foreign to the host vector.

More preferably the heterologous nucleotide sequence is associated with promoter/leader and poly A sequences.

In one method of construction of a suitable vector the non-essential region to be altered to incorporate foreign DNA could be constructed via homologous recombination. By this method the non-essential region is cloned and foreign DNA together with promoter, leader and poly adenylation sequences is inserted preferably by homologous recombination between flanking sequences. By this method also, deletion of portions of the non-essential region is possible to create extra room for larger DNA inserts that are beyond the normal packing constraints of the virus.

By this method a DNA expression cassette containing an appropriate PAV promoter with foreign gene sequence as well as leader sequences and poly adenylation recognition sequences can be constructed with the unique restriction enzyme sites flanking the cassette enabling easy insertion into the

after a period of 4 weeks subsequent to initial vaccination.

In a further embodiment of the invention there is provided a method for producing an immune response in a pig including administering to the pig an effective amount of a recombinant vaccine according to the invention. An effective amount is an amount sufficient to elicit an immune response, preferably at least 10^4 $TCID_{50}$ per dose.

The vaccine of the invention may of course be combined with vaccines against other viruses or organisms such as parvovirus or Aujeszky's disease at the time of its administration.

In a preferred aspect of this embodiment of the invention, administration is by oral delivery or intra-nasally.

Methods for construction and testing of recombinant vectors and vaccines according to this invention will be well known to those skilled in the art. Standard procedures for endonuclease digestion, ligation and electrophoresis were carried out in accordance with the manufacturer's or suppliers instructions. Standard techniques are not described in detail and will be well understood by persons skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the DNA restriction endonuclease map of the entire PAV serotype 3 genome.

Figure 2 illustrates the sequence characterisation and cloning of the major later promoter and splice leader sequences of PAV serotype 3.

Figure 3 illustrates the sequences of the major later promoter, upstream enhancer sequence and splice leaders 1, 2 and 3.

Figure 4 illustrates the terminal 720 bases of the right end of the genome.

Figure 5 illustrates the promoter region of E3 and the overlapping L4 area.

Figure 6 illustrates a preferred method of construction of a PAV vector.

Figure 7 represents temperature data of pigs vaccinated with a PAV based vaccine following challenge with CSFV antigen.

Figure 8 graphically represents anti-PAV antibody levels detected by ELISA in pigs pre and post vaccination with a PAV based vaccine.

by experimental infection (Edington *et al.*, 1972). Experimental infections with gastrointestinal serotypes of the virus (eg serotype 3) have been able to produce diarrhoea but the pathological changes produced were not clinically significant.

The genome of the selected PAV serotype 3 was characterised by conventional methods. The DNA restriction endonuclease maps of the entire genome is illustrated in figure 1. The genomes are orientated left to right. By convention adenovirus genomes are normally orientated such that the terminal region from which no late mRNA transcripts are synthesised is located at the left end. The enzymes used to generate the map are indicated at the edge of each map.

CHARACTERISATION OF MAJOR LATE PROMOTER (MLP) AND SPLICE LEADER SEQUENCES (LS) OF PAV SEROTYPE 3

Identification and cloning of the PAV MLP

By use of restriction enzyme and genetic maps of the PAV serotype 3 genome, a region was located that contained the MLP and leader sequences (Fig 1). The fragments identified in this region were cloned into plasmid vectors and sequenced.

The MLP promoter sequence was identified as containing a classical TATA sequence, the only one in the region sequenced, as well as upstream factors and was subsequently confirmed by the location of the leader sequence and the transcriptional start site.

Figures 2 and 3 illustrate the sequence characterisation of the major late promoter and splice leader sequences of PAV serotype 3.

In order to determine the structure and sequence of the leader sequence spliced to late mRNA, porcine kidney cells were infected with PAV and the infection was allowed to proceed until late in the infection cycle (usually 20-24 hr p.i.). At this time total RNA was purified from the infected cells using the RNAgents total RNA purification kit (Promega). The isolated RNA was precipitated with isopropanol and stored at -70°C in 200 µl aliquots until

CHARACTERISATION OF NON-ESSENTIAL REGIONS OF VIRAL GENOME

The right end was identified by cloning and complete sequencing of the PAV serotype 3 *Apa*I fragment J of approximately 1.8 Kbp. The inverted terminal repeat (ITR) has been determined by comparison of the RHE sequence with that of the left hand end. The ITR is 144 bases long and represents the starting point into which potential insertions can be made. Figure 4 shows the sequence of the terminal 720 bases. Restriction endonuclease sites of interest for insertion of foreign DNA are indicated in the terminal sequence. A putative TATA site for the E4 promoter is identified, this being the left most end for the possible site of insertion. Initial insertions will be made into the *Sma*I or *Eco*RI sites.

The E3 region of the genome, this also being a non-essential area, has been located and cloned. The promoter region of E3 has been identified and the overlapping L4 area sequenced (Figure 5). The region of the E3 after the polyadenylation signal of the L4 is also a possible site for insertion and can also be used for deletion to create more room for larger cassette insertions.

CONSTRUCTION OF PAV VECTOR

Figure 6 illustrates a preferred method of construction of a PAV vector. The right hand end *Apa*I fragment J of PAV serotype 3 is cloned and a unique *Sma*I restriction endonuclease site 230 bp from the inverted repeats was used as an insertion site.

The major late promoter expression cassette containing the E2 (gp55) gene of classical swine fever virus (hog cholera virus) was cloned into the *Sma*I site of the RHE fragment.

A preferred method of homologous recombination was cutting genomic PAV 3 DNA with *Hpa*I, a unique site in the genome, and transfecting this DNA with *Apa*I cut expression cassette plasmid containing gp55.

The DNA mix was transfected into preferably primary pig kidney cells by standard calcium chloride precipitation techniques.

The preferred method of transfection generates recombinant virus through homologous recombination between genomic PAV 3 and plasmid (Fig 6).

2 Construction of recombinant PAV-G-CSF

An expression cassette comprising of the porcine adenovirus major late promoter, the gene encoding porcine granulocyte-colony stimulating factor (G-CSF) and SV40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 1.28 kilobase pairs. No deletion of the genomic PAV 3 was made. The recombinant virus was plaque purified two times and passaged stably in primary pig kidney cells. The recombinant was shown to contain G-CSF by Southern blot hybridisation and polymerase chain reaction (PCR). Expression of G-CSF was demonstrated by infecting primary kidney cells with the recombinant PAV-G-CSF. Tissue culture supernatants from the infected primary kidney cells were then electrophoresed in SDS-PAGE gels and transferred to filters. Infected cells expressing G-CSF were detected in a Western blot using a rabbit polyclonal antiserum against porcine G-CSF expressed by purified recombinant *E coli*.

3 Construction of recombinant PAV-gp55T/GM-CSF

An expression cassette consisting of the porcine adenovirus major late promoter, a truncated form of the classical swine fever virus gene gp55 fused in frame to the gene encoding either the full length or the mature form of porcine granulocyte/macrophage-colony stimulating factor (GM-CSF) and SV40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 2.1 kilobase pairs. No deletion of the genomic PAV 3 was made. The recombinant virus was plaque purified two times and shown to contain gp55 and GM-CSF by PCR.

4 Construction of recombinant PAV-gp55/E3

An expression cassette consisting of the porcine adenovirus major late promoter, the classical swine fever virus gene gp55 and SV40 polyA was inserted between the *BsrgI/SnaBI* sites in the E3 region (MU 81-84) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a

Table 2 Temperatures post challenge with CSFV (°C)

Pig No.	Day -1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2		39.6	39.9	40.1	40.3	40.1	39.2	39.7	39.5	39.5	3	9					
7	39.4	39.1	39.5	39.1	40.0	39.4	38.9										
3		39.6	40.4	39.6	40.0	40.7	40.7	41.9	40.7	40.9	42.0+						
6		39.5	39.5	40.0	40.0	39.6	40.5	39.9	39.2	39.2	3	8					
8	39.3	38.8	38.9	39.6	39.3	39.1	38.9										
7		39.8	39.9	40.4	40.6	40.3	39.7	39.7	39.7	39.5	3	9					
3	39.1	39.3	39.6	40.6	39.7	39.8	39.7										
8		39.9	40.6	40.5	40.3	40.0	41.4	39.8	41.0	40.6	39.0+						
11		39.6	39.9	40.0	40.3	40.7	40.5	40.0	41.8	41.5	41.3+						
12		39.8	39.9	40.9	41.0	41.2	40.6	40.1	41.0	41.7	40.3+						
13		39.7	40.0	41.2	41.5	41.6	41.0	39.7+									
14		39.3	40.0	39.6	39.8	40.3	40.7	41.2	40.8	40.2	41.7+						

mean white blood cell (WBC) counts for each group monitored. These results are graphically represented in Figure 10 and the percentage change in mean WBC counts graphically represented in Figure 11.

Pigs vaccinated with either PAV wt or PAV-G-CSF showed clinical signs of disease with mild diahorrea 24-72 hours post vaccination. Both groups of pigs were completely recovered by 80-96 hours post-vaccination. Control pigs showed no clinical signs of disease.

Complete blood screening results show that the mean WBC counts for control pigs increased over the duration of the experiment.

PAV wt vaccinated pigs also show an increase in WBC counts, with a depression in WBC counts between 48-80 hours post-vaccination and recovery from 80-96 hours onwards.

Pigs vaccinated with the recombinant PAV-G-CSF show a significant depression in WBC counts over the duration of the experiment. A statistical analysis of these results is tabulated in Table 3. The analysis shows that differences between the mean WBC counts (controls and PAV-G-CSF; PAV wt and PAV-G-CSF) were significant indicating that the recombinant PAV-G-CSF altered the proportions of cells involved with immunity.

Table 3. Results of t-tests between mean WBC counts of groups of pigs vaccinated with either PAV wild type (wt), PAV recombinant expressing G-CSF (PAV-G-CSF) or unvaccinated controls.

	Pre vacc O hr	8-24 hr[d]	32-48 hr	56-72 hr	80-104 hr
Control vs PAV-G-CSF[a]	p> 0.2[b]	p> 0.2	p> 0.2	p> 0.2	p< 0.005
Control vs PAV wt	p> 0.1	p> 0.01[c]	p> 0.02	p> 0.2	p< 0.05
PAV-G-CSF vs PAV wt	p> 0.2	p< 0.05	p< 0.05	p< 0.05	p< 0.001

a: null hypothesis; there is no difference between the mean WBC counts.

b: p>0.05, insufficient to reject the null hypotheses at the 95% confidence level, conclude that there is no difference between mean leucocyte levels.

c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean leucocyte levels.

d: 4 pigs in each group were bled at 8 hour intervals.

Table 5. Results of t-tests between mean lymphocyte cell populations following vaccination of pigs with either recombinant PAV-G-CSF, wild type PAV (PAV wt) or unvaccinated controls.

	pre vacc	8-24hr[d]	32-48hr	56-72hr	80-96hr	104hr
conrols vs PAV-G-CSF[a]	p>0.2	p>0.05[b]	p>0.2	p>0.2	p>0.2	p>0.2
controls vsPAV wt	p>0.2	p>0.2	p<0.01[c]	p<0.001[c]	p<0.001[c]	p>0.2
PAV wt vs PAV-G-CSF	p>0.2	p<0.05[c]	p<0.002[c]	p<0.005[c]	p<0.001[c]	p>0.05

a: null hypothesis; there is no difference between the mean lymphocyte cell counts.

b: p>0.05, insufficient to reject the null hypothesis at the 95% confidence level, conclude that there is no difference between mean lymphocyte cell levels.

c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean lymphocyte cell levels.

d: 4 pigs in each group were bled at 8 hour intervals

Figure 14 graphically represents changes in the proliferation of T cells of each group following stimulation with Concanavalin A (Con A). These results confirm that there was a significant proliferation of T-cells following vaccination with PAV wt at day 2 post vaccination, whereas vaccination with the recombinant PAV-G-CSF resulted in a suppression of T-cell proliferation by day 3.

The results of vaccination with a recombinant PAV expressing porcine G–CSF shows that G-CSF has a significant effect on the cells involved with immune responses.

It will be appreciated that whilst this document establishes the metes and bounds of this invention, all embodiments falling within its scope for example with regard to heterologous genes, insertion sites, types of promoter and serotype have not necessarily been specifically exemplified although it is intended that they should fall within the scope of protection afforded this invention.

Figure 3

Individual sequences of the Promoter cassette components:

I. The 5' (upstream) sequence included in the long cassette.

```
  1   GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCAGTCG GAGATGAAGG CACGCGCCCA
 61   GGCGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCG TTGGGCACTA ATGGCGAGGC
121   CTGCTCGAGC GTGTGGAGAC AGAGGTCCTC GTCGTCCGCG TCCAGGAAGT GGATTGGTCG
181   CCAGTGGTAG
```

II. Sequence including the USF, TATA motiff and sequence to the cap site.

```
  1   CCACGTGACC GGCTTGCGGG TCGGGGGGTA TAAAAGGCGC GGGCCGGGGT GCGTGGCCGT
 61   C
```

III. First leader sequence.

```
  1   AGTTGCTTCG CAGGCCTCGT CACCGGAGTC CGCGTCTCCG GCGTCTCGCG CTGCGGCTGC
 61   ATCTGTGGTC CCGGAGTCTT CAG
```

IV. Second leader sequence.

```
  1   GTCCTTGTTG AGGAGGTACT CCTGATCGCT GTCCCAGTAC TTGGCGTGTG GGAAGCCGTC
 61   CTGATCG
```

V. Third leader sequence.

```
  1   CGATCCTCCT GCTGTTGCAG CGCTTCGGCA AACACGCGCA CCTGCTCTTC GGACCCGGCG
 61   AAGCGTTCGA CGAAGGCGTC TAGCCAGCAA CAGTCGCAAG
```

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant porcine adenovirus capable of expressing DNA of interest, said DNA of interest being stably integrated into an appropriate site of said recombinant porcine adenovirus genome.

2. A recombinant vector including a recombinant porcine adenovirus stably incorporating, and capable of expressing DNA of interest.

3. A recombinant vector as claimed in claim 2 wherein said recombinant porcine adenovirus is capable of expression of at least one heterologous nucleotide sequence.

4. A recombinant vector as claimed in claims 2 or 3 wherein said recombinant porcine adenovirus includes a live porcine adenovirus having virion structural proteins unchanged from those in a native porcine adenovirus from which said recombinant porcine adenovirus is derived.

5. A recombinant vector as claimed in claims 3 or 4 wherein said at least one heterologous nucleotide sequence is capable of expression as an antigenic polypeptide.

6 A recombinant vector as claimed in claims 3 or 4 wherein said at least one heterologous nucleotide sequence is capable of expression as an immuno-potentiating molecule.

7 A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes antigenic determinants of infectious agents causing intestinal diseases in pigs.

17. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of Mycoplasma hyopneumonia.

18. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes FLT-3 ligand.

19. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes interleukin 3 (IL-3).

20. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine interleukin 4 (IL4).

21. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes gamma interferon (γIFN).

22. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine granulocyte macrophage colony stimulating factor (GM-CSF).

23. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine granulocyte colony stimulating factor (G-CSF).

24. A recombinant vector as claimed in claims 3 or 4 wherein said heterologous nucleotide sequence encodes an antigenic polypeptide and an immuno-potentiating molecule.

25. A recombinant vector as claimed in any one of claims 2 to 24 wherein said recombinant porcine adenovirus is selected from the group consisting of serotypes 3 and 4.

34. A recombinant vaccine as claimed in claim 33 wherein the said at least one heterologous nucleotide sequence is capable of expression as an antigenic polypeptide.

35. A recombinant vaccine as claimed in claim 33 wherein said at least one heterologous nucleotide sequence is capable of expression as an immuno-potentiating molecule.

36. A recombinant vaccine as claimed in claim 33 wherein said heterologous nucleotide sequence encodes an antigenic polypeptide and an immuno-potentiating molecule.

37. A recombinant vaccine as claimed in any one of claims 33 to 36 wherein said carriers and/or excipients are selected such that said vaccine is deliverable in the form of an aerosol spray, an enteric coated dosage unit or an inoculum.

38. A method of producing a recombinant vaccine as claimed in any one of claims 33 to 36 including admixing at least one recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence together with suitable carriers and/or excipients.

39. A method of vaccination of pigs against disease including administering to said pigs a first recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence encoding an antigenic determinant of said disease against which vaccination is desired.

40. A method as claimed in claim 39 including administering to said pig a second porcine adenovirus vector including at least one heterologous nucleotide sequence which differs from said at least one heterologous nucleotide sequence incorporated in said first recombinant porcine adenovirus vector.

Fig 1.

Restriction enzyme maps of the PAV3 grnome

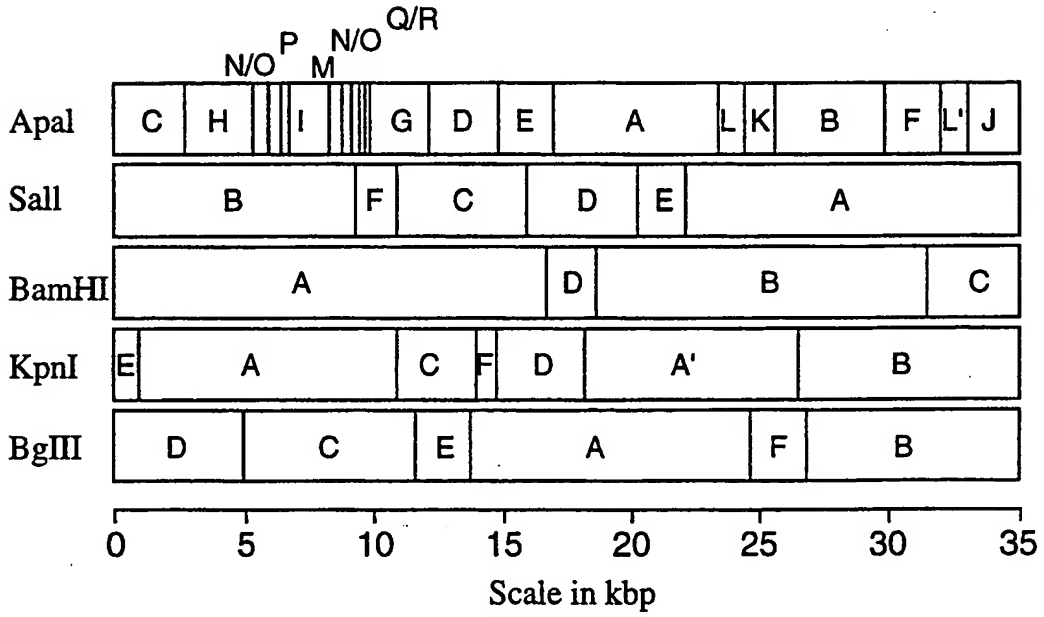

Fig 3.

Individual sequences of the Promoter cassette components:

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```
   1   GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCAGTCG GAGATGAAGG CACGCGCCCA
  61   GGCGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCG TTGGGCACTA ATGGCGAGGC
 121   CTGCTCGAGC GTGTGGAGAC AGAGGTCCTC GTCGTCCGCG TCCAGGAAGT GGATTGGTCG
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II. Sequence including the USF, TATA motiff and sequence to the cap site.

```
   1   CCACGTGACC GGCTTGCGGG TCGGGGGGTA TAAAAGGCGC GGGCCGGGGT GCGTGGCCGT
  61   C
```

III. First leader sequence.

```
   1   AGTTGCTTCG CAGGCCTCGT CACCGGAGTC CGCGTCTCCG GCGTCTCGCG CTGCGGCTGC
  61   ATCTGTGGTC CCGGAGTCTT CAG
```

IV. Second leader sequence.

```
   1   GTCCTTGTTG AGGAGGTACT CCTGATCGCT GTCCCAGTAC TTGGCGTGTG GGAAGCCGTC
  61   CTGATCG
```

V. Third leader sequence.

```
   1   CGATCCTCCT GCTGTTGCAG CGCTTCGGCA AACACGCGCA CCTGCTCTTC GGACCCGGCG
  61   AAGCGTTCGA CGAAGGCGTC TAGCCAGCAA CAGTCGCAAG
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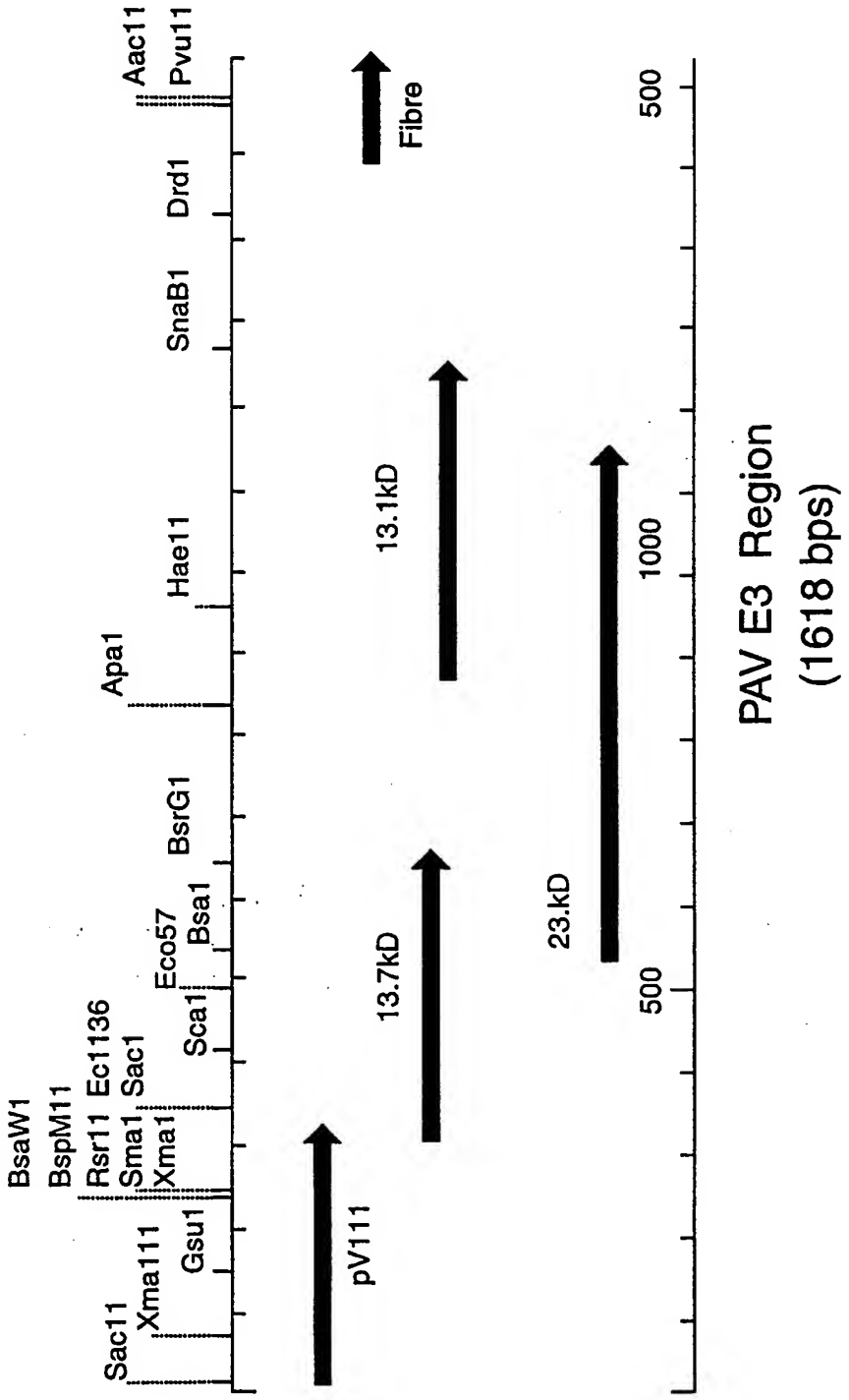
Aac11
Pvu11
Drd1
SnaB1
Hae11
Apa1
BsrG1
Bsa1
Eco57
Sca1
Sac1
Ec1136
Rsr11
BspM11
BsaW1
Sma1
Xma1
Gsu1
Xma111
Sac11
Fibre
13.1kD
13.7kD
23.kD
pV111
500
1000
500
PAV E3 Region
(1618 bps)

Fig 5.

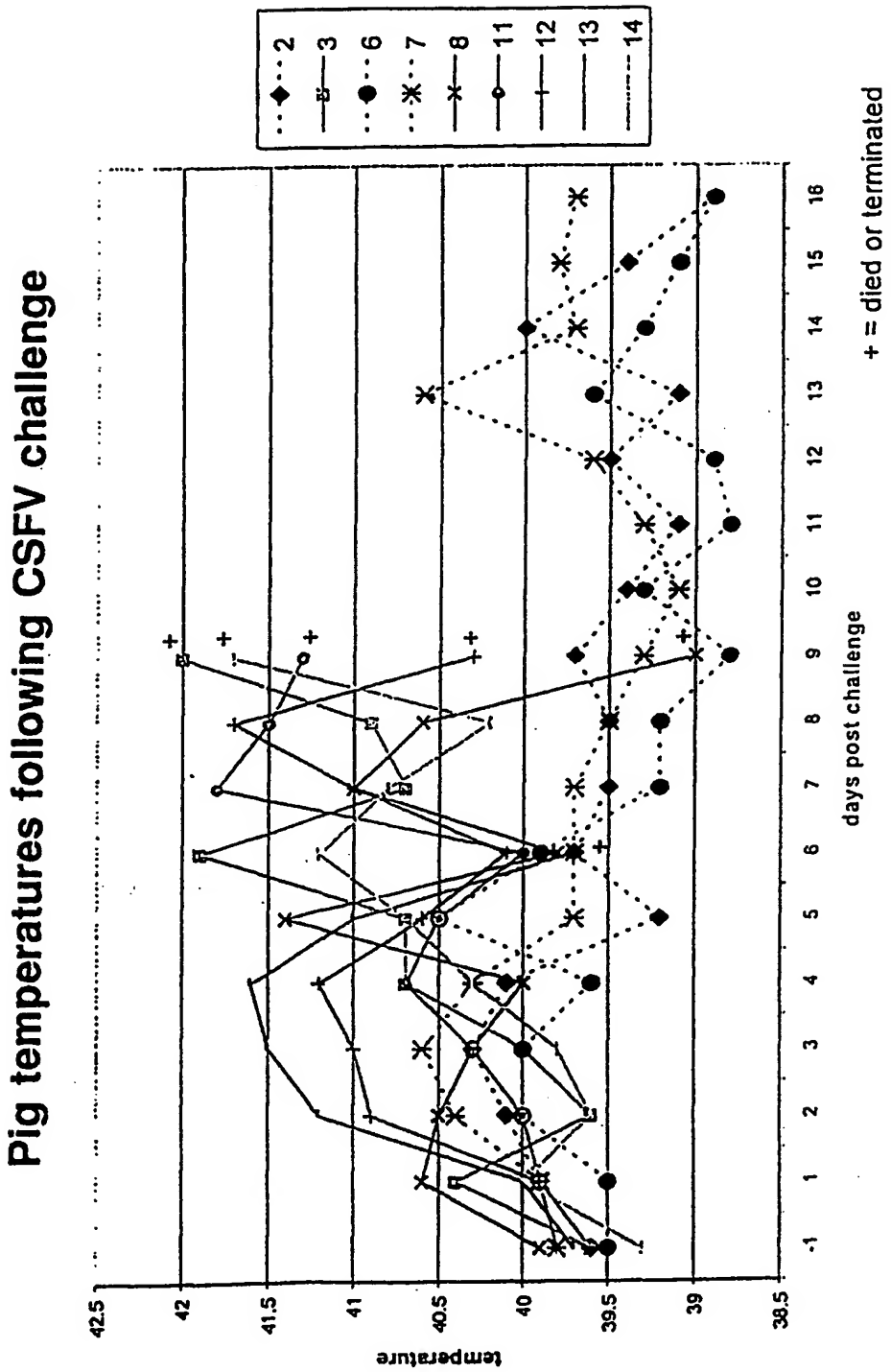
Pig temperatures following CSFV challenge
2
3
6
7
8
11
12
13
14
temperature
42.5
42
41.5
41
40.5
40
39.5
39
38.5
days post challenge
-1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
+ = died or terminated

Fig 7.

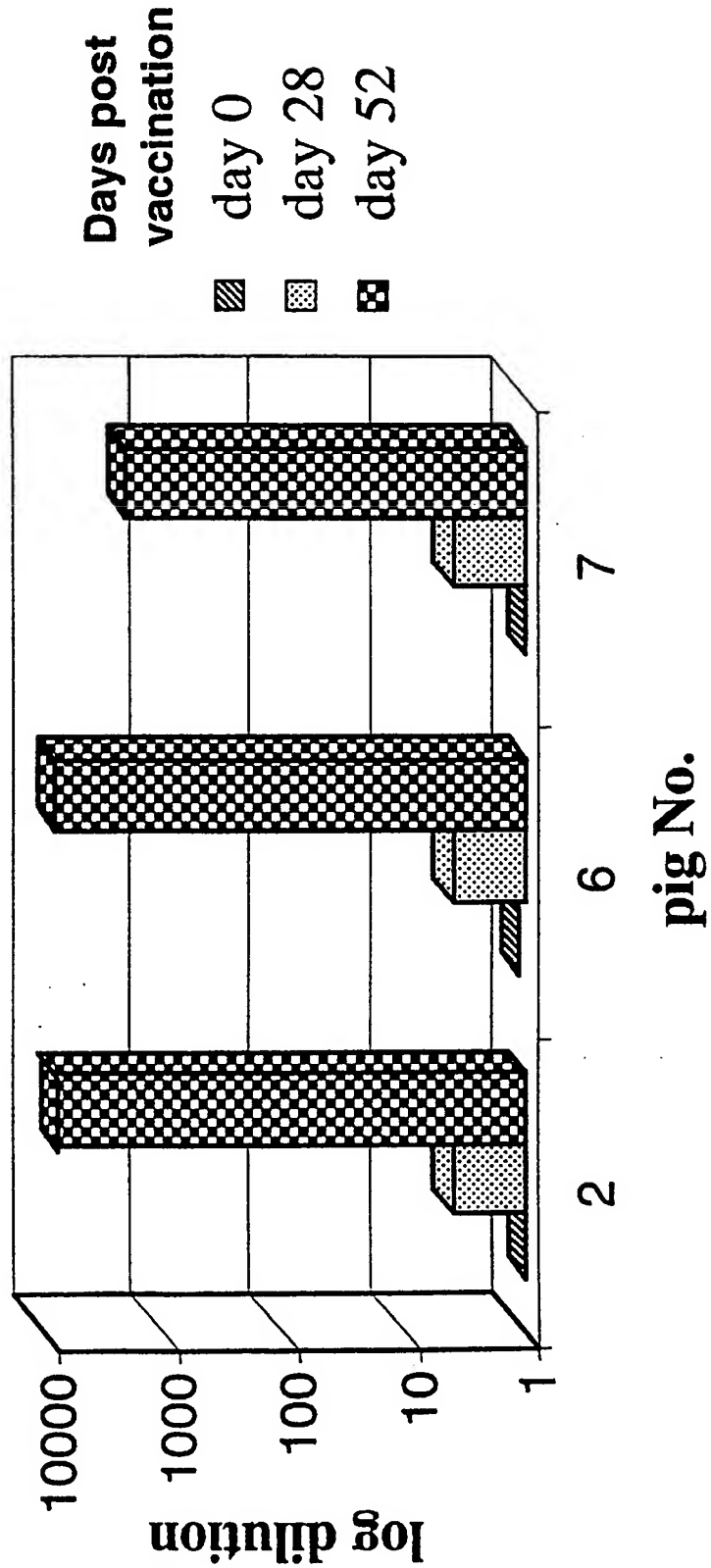
CSFV SN Ab titres in pig sera
Days post vaccination
day 0
day 28
day 52
log dilution
10000
1000
100
10
1
2
6
7
pig No.

Fig 9.

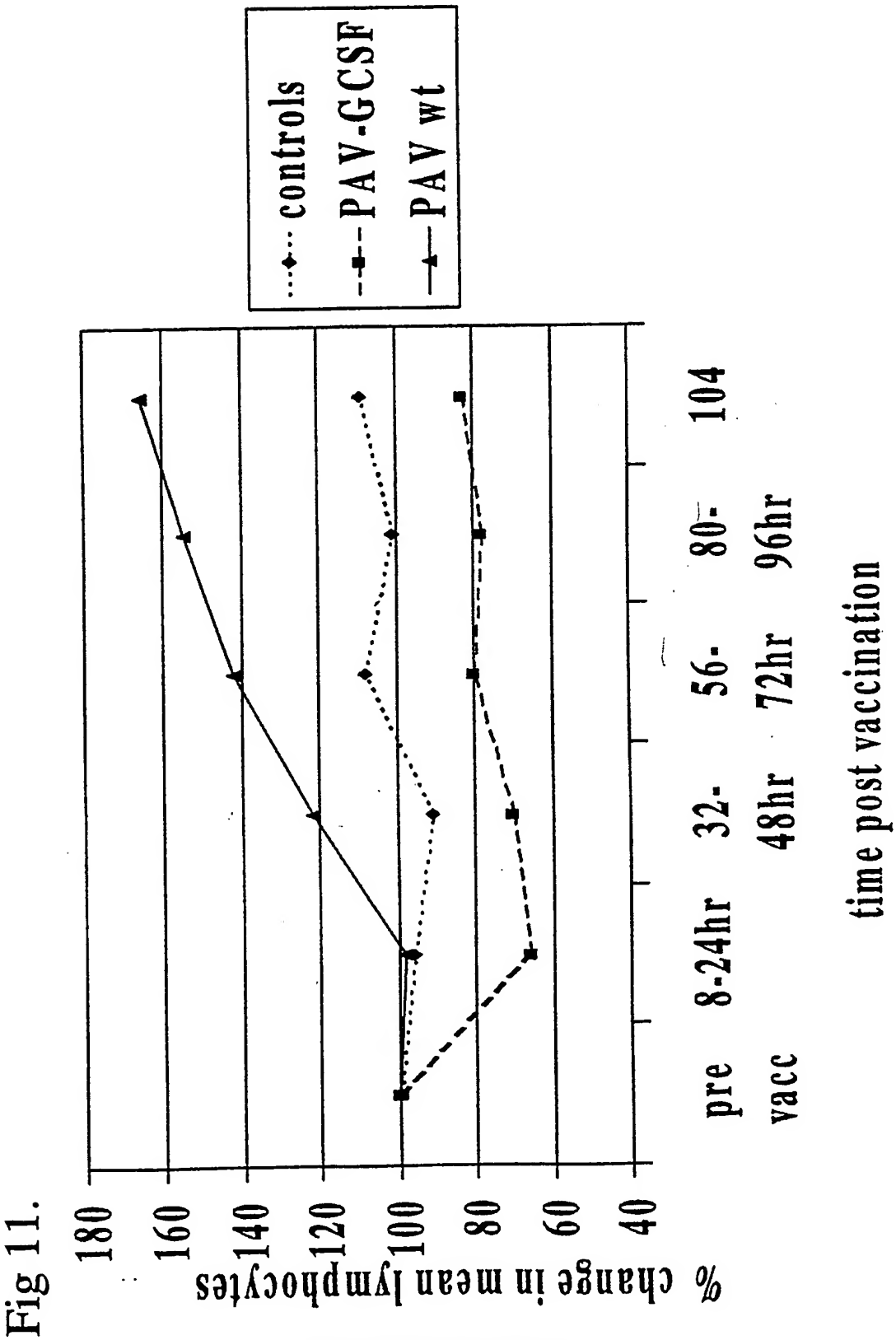
controls
PAV-GCSF
PAV wt
% change in mean lymphocytes
180
160
140
120
100
80
60
40
pre vacc
8-24hr
32-48hr
56-72hr
80-96hr
104
time post vaccination

Fig 11.

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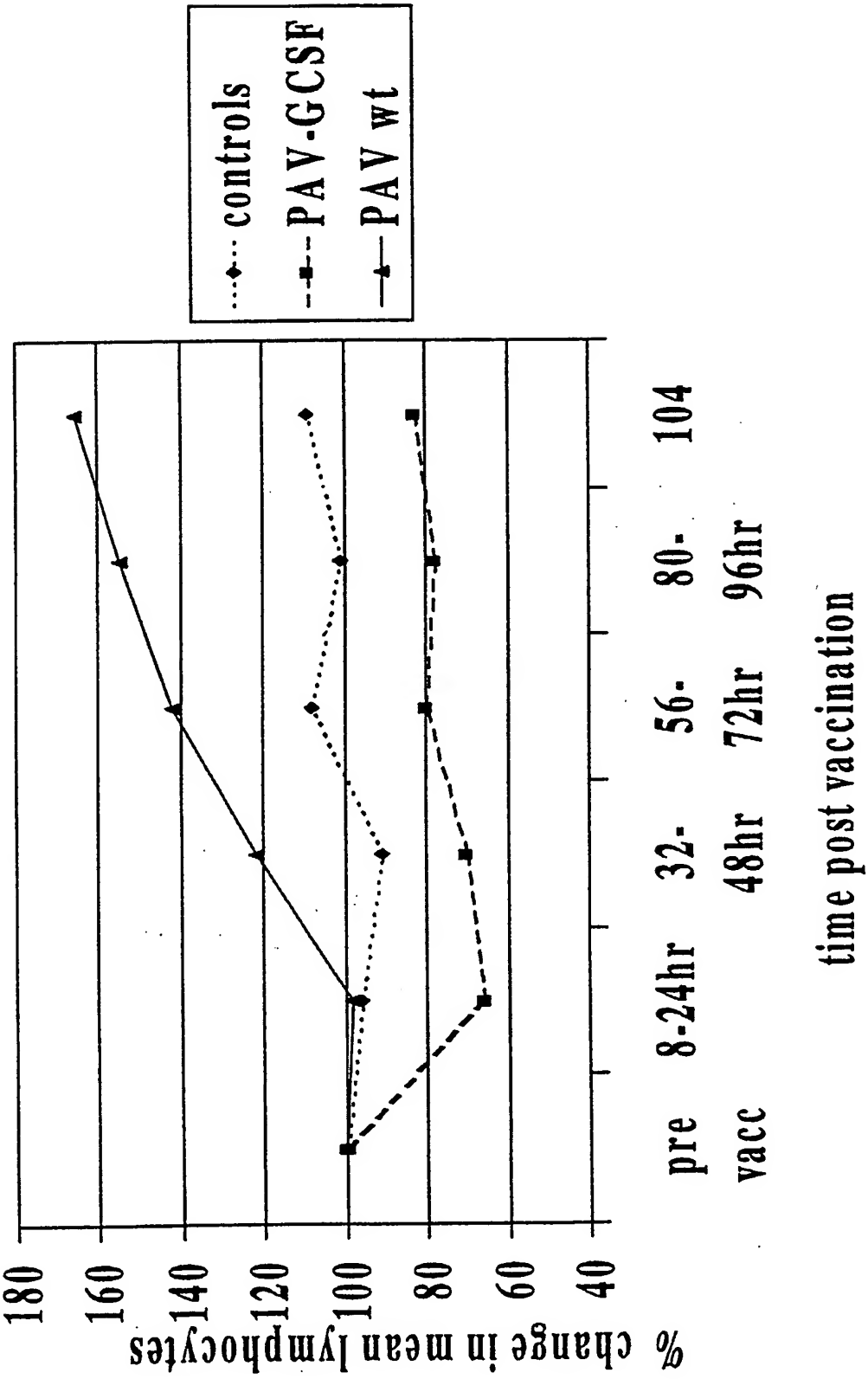
controls
PAV-GCSF
PAV wt
% change in mean lymphocytes
180
160
140
120
100
80
60
40
pre vacc
8-24hr
32-48hr
56-72hr
80-96hr
104
time post vaccination

Fig 13.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/00648

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61K 039/235 ; C12N 15/63, 15/67. 15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Derwent Database - WPAT ; Chemical Abstracts - Keywords below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Derwent Database - JAPIO, USPM; Medline - Keywords below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, JAPIO, USPM: Keywords- (swine# or porcine# or hog# or pig#) (20N) adenovir:
Chemical Abstracts, Medline: Keywords- adenovir? (20N) (swine or porcine or hog or pig)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Derwent Abstract Accession Number: 97-310593 (WO 97/20036 (CYANAMID IBERICA) publ. 5 June 1997) (See examples)	1-4, 25-27, 31-33, 39 5-24, 34-38, 40-42
Y	AU, A, 72646/94 (RHONE POULENC RORER) OPI. 13 February 1995 (see page 2, Examples, Claims 9 and 10)	1-27, 31-42
X Y	Advances in Experimental Medicine and Biology, Vol. 342, 1993, P. Callebaut et al. "Construction of a Recombinant Adenovirus for the Expression of the Glycoprotein S Antigen of Porcine Respiratory Coronaviruses" pp 469- 470 (see entire document)	1-4, 25-27, 31-33, 39 5-24, 34-38, 40-42

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" Document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 October 1998	16 OCT 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer KAREN TAN Telephone No.: (02) 6283

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 98/00648

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	97/20036	AU	71325/96	ES	1032067	ES	2105984
AU	72646/94	WO	95/02697	EP	667912	FI	951138
		NO	950939	SK	312/95	AU	69052/98
		CA	2144040	CN	1113390	CZ	9500639
		FR	2718749	HU	72558	NZ	269156
		PL	308122				

END OF ANNEX